

ORIGINAL RESEARCH ARTICLE

Statin Regulated ERK5 Stimulates Tight Junction Formation and Reduces Permeability in Human Cardiac Endothelial Cells[†]

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Abstract

The MEKK3/MEK5/ERK5 signalling axis is required for cardiovascular development *in vivo*. We analysed the physiological role of ERK5 in cardiac endothelial cells and the consequence of activation of this kinase by the statin class of HMG Co-A reductase inhibitor drugs. We utilised human microvascular endothelial cells (HCMECs) and altered ERK5 expression using siRNA mediated gene silencing or overexpression of constitutively active MEK5 and ERK5 to reveal a role for ERK5 in regulating endothelial tight junction formation and cell permeability. Statin treatment of HCMECs stimulated activation of ERK5 and translocation to the plasma membrane resulting in co-localisation with the tight junction protein ZO-1 and a concomitant reduction in endothelial cell permeability. Statin mediated activation of ERK5 was a consequence of reduced isoprenoid synthesis following HMG Co-A reductase inhibition. Statin pretreatment could overcome the effect of doxorubicin in reducing endothelial tight junction formation and prevent increased permeability. Our data provides the first evidence for the role of ERK5 in regulating endothelial tight junction formation and endothelial cell permeability. Statin mediated ERK5 activation and the resulting decrease in cardiac endothelial cell permeability may contribute to the cardioprotective effects of statins in reducing doxorubicin-induced cardiotoxicity. This article is protected by copyright. All rights reserved

Keywords: ERK5, statin, ZO-1, permeability, cardiotoxicity

Introduction

Endothelial cells form a monolayer on the luminal surface of the vasculature providing an interface that regulates the transcellular and paracellular passage of solutes and xenobiotics between circulation and tissues. Endothelial cell survival and proliferation is regulated by mitogen activated protein kinases (MAPKs) (Nithianandarajah-Jones et al., 2012; Nithianandarajah-Jones et al., 2014). There are 4 major MAPKs: ERK1/2, JNK, P38 and ERK5. ERK5 is the least studied of the 4 major MAPKs, sharing 66% sequence homology with ERK2. However, ERK5 differs from other MAPKs with its extended C terminal tail containing a number of phosphorylation sites and transcriptional activation domain (Buschbeck and Ullrich, 2005). The ERK5 signalling axis has been demonstrated to be essential for cardiac development; knockout of *MEKK3/MEK5/ERK5* in mice is embryonically lethal at E9.5 - 10.5 (Hayashi et al., 2004; Wang et al., 2005; Yang et al., 2000). Importantly, knockout of *ERK5* in adult mice leads to increased vascular leakage ultimately leading to death within 2-4 weeks (Hayashi et al., 2004). This data shows that ERK5 activity is required in adults to preserve vascular integrity.

Endothelial junctions regulate the paracellular passage of ions and xenobiotics from the circulation to underlying tissues (Gonzalez-Mariscal et al., 2005). Endothelial cells express tight, adherens and gap junctions (Dejana et al., 1995). These have been demonstrated to vary in isoform expression depending on their anatomical location. Tight junctions located within the brain microvasculature contain significantly higher levels of claudin 5 than in the glomerular vasculature, reflecting the difference in permeability between the vascular beds (Gunzel and Yu, 2013). Zonula occludens-1 (ZO-1) is a tight junction protein, which has recently been shown to regulate adherens junctions, angiogenesis and endothelial barrier formation (Tornavaca et al., 2015). The endothelial barrier is altered in several diseases including peripheral oedema (Dong et al., 2016), stroke, multiple sclerosis and neurodegenerative diseases (Shi et al., 2016). In these conditions, changes in endothelial permeability allow water and solutes to permeate to underlying tissues, which leads to infiltration of immune cells (Shi et al., 2016). It is becoming apparent that anti-cancer drugs are able to induce changes in endothelial permeability, which could be an initiating event in drug-induced cardiotoxicity (Cross et al., 2015; Dong et al., 2016; Wolf and Baynes, 2006). We have recently shown that the anti-cancer drugs doxorubicin and trastuzumab (Herceptin) can adversely affect cardiac microvascular endothelial tight junction formation leading to increased drug permeability (Wilkinson et al., 2016).

ERK5 has been shown to be activated by a number of growth factors in a range of cell types (Nithianandarajah-Jones et al., 2012). We have previously shown that in endothelial cells, VEGF stimulates ERK5 activity leading to increased cell survival (Roberts et al., 2010). Recent data has shown that vasoprotective drugs such as statins,

which inhibit 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase) can also activate ERK5 in endothelial cells (Ohnesorge et al., 2010). In addition to their ability to lower LDL-cholesterol, statins have several pleiotropic effects such as cardioprotection, however the mechanism behind this is not yet fully understood (Chen et al., 2013; Henninger et al., 2012; Wang et al., 2008). Statins competitively inhibit HMG-CoA reductase (Stancu and Sima, 2001) preventing the production of cholesterol as well as isoprenoid intermediates such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphates (GGPP) (Sen-Banerjee et al., 2005). The ability of statins to ameliorate drug-induced cardiovascular toxicity has recently been demonstrated in animal models, with cardioprotective effects attributed to effects on cardiomyocytes (Henninger et al., 2015; Yoshida et al., 2009).

Here we report that statins activated ERK5 in human cardiac microvascular endothelial cells and that this activation results in an increase in tight junction formation and decreased permeability; statins can ultimately protect against doxorubicin injury of endothelial cells, which may contribute to the cardioprotective effects of these drugs.

Materials and Methods

Cell culture

Human cardiac microvascular endothelial cells (HCMECs, C12285), human dermal microvascular endothelial cells (HDMECs, C-12212), human coronary artery endothelial cells (HCAECs, C-12221), human brain microvascular endothelial cells (HBMECs, C-12287), human umbilical vein endothelial cells (HUVECs, C-12205) were purchased from PromoCell (Heidelberg, Germany) and cultured as described previously (Wilkinson et al., 2016). A2780 cells were routinely cultured in RPMI 1640 medium (61870044, Gibco) supplemented with 10% (v/v) FCS. BT474 cells were routinely cultured in DMEM medium (D6429, Sigma, Poole, UK) supplemented with 10% (v/v) FCS. Cells were incubated at 37°C in humidified air containing 5% (v/v) CO₂.

Cell stimulation and preparation of cell lysates

HCMECs were grown to confluence over 6 days in FGM with changes every 2-3 days, before addition of drugs. Simvastatin (S6196 purchased from Selleck Chemicals) was diluted to 0.3 µM in EBM supplemented with 1% (v/v) FCS and then added to cells for 6hrs either alone or as a pre-incubation before addition of doxorubicin (S1208, Selleck Chemicals) diluted to 0.1 µM for a further 6hrs. BIX02189 (S1531, Selleck Chemicals) diluted to 1 µM was added 30 minutes prior to simvastatin treatment.

Cells were washed in ice-cold DPBS on ice followed by addition of RIPA (20 mM Tris pH 7.5, 150 mM NaCl, 2.5 mM EDTA, 10% (w/v) glycerol, 1% (v/v) Triton X-100; 1 mM Na₃VO₄; 10 µg/ml Aprotinin; 10 µg/ml Leupeptin; 10 µg/ml Pepstatin A; 1 mM PMSF; 0.5% (v/v) SDS and 0.5% (v/v) sodium deoxycholate) lysis buffer. Lysates were centrifuged [17,000 x g for 20 minutes at 4°C] before diluting in LDS sample buffer (Invitrogen, Paisley, UK) containing 2-mercaptoethanol (Sigma, Poole, UK) and denaturing at 90°C for 5 minutes.

Cell Fractionation

Cellular fraction was performed using a ThermoScientific subcellular protein fractionation kit (78840). In brief, cells were harvested with trypsin-EDTA and centrifuged [500 x g for 5 minutes]. All buffers contain the protease inhibitor cocktail provided with the kit. The cell pellet was washed in ice cold PBS and centrifuged [500 x g for 5 minutes]. The cell pellet was resuspended in cytoplasmic extraction buffer (CEB) and incubated on ice for 10 minutes. Lysates were centrifuged [500 x g for 5 minutes] and the supernatant transferred to a new tube for the cytoplasmic fraction. The pellet was resuspended in membrane extraction buffer (MEB) and incubated on ice for 10 minutes. Lysates were centrifuged [3000 x g for 5 minutes] and the supernatant transferred to a new tube for

the membrane fraction. The pellet was resuspended in nuclear extraction buffer (NEB) and incubated on ice for 30 minutes. Lysates were centrifuged [5000 \times g for 5 minutes] and the supernatant transferred to a new tube as the nuclear fraction. All fractions were diluted with LDS sample buffer containing 2-mercaptoethanol and denatured at 90°C for 5 minutes.

Western Blot and Immunoprecipitation

For immunoprecipitation, lysates were incubated with ERK5 (2 μ g/mL, #AF2848, R&D systems) or control IgG (2 μ g/mL, goat) and protein G agarose. Lysates were separated by SDS-PAGE on 8% Tris-glycine gels (8% (w/v) acrylamide, 0.4 M Tris-HCl pH8.8 0.08% (w/v) SDS, 6.2% (v/v) glycerol 0.05% (v/v) TEMED and 0.02% (w/v) APS). Gels were resolved for 90 minutes at 35 mA and transferred to 0.2 μ m nitrocellulose membrane (Hybind-C, GE Healthcare, Amersham, UK) for 2hrs at 125 mA. Membranes were blocked in 5% (w/v) bovine serum albumin (BSA) diluted in Tris-buffered saline (TBS) with 0.1% (v/v) Tween- 20 (TBST) and probed with primary antibodies, directed against: ERK5 (#3372), phospho ERK1/2 (#4370), GAPDH (#5174), phospho ERK5 (T218/Y220) (#3371), ERK1/2 (#4695) were purchased from New England Biolabs (Hitchin, UK). Antibodies to RAP1 (SC-65) and unphosphorylated RAP1A (SC-1482) were purchased from Santa Cruz Biotechnology. Antibodies to MEKK2 (ab33918), MEKK3 (ab40750) and MEK5 (ab45146) were purchased from Abcam (Cambridge, UK). Antibody to ZO-1 (40-2200) was purchased from Invitrogen (Thermo Fisher Scientific). All antibodies were diluted in 1% (w/v) BSA and incubated overnight at 4°C. Proteins were detected using rabbit- or goat- specific HRP secondary antibodies (Jackson ImmunoResearch Labs) and enhanced-chemiluminescence (ECL) western blotting detection reagent (Pierce).

Immunofluorescence

HCMECs were fixed in 2% (w/v) PFA, permeabilised in 0.25% (v/v) triton X-100 (for ZO-1 and Alexa Fluor® Phalloidin 568 staining). For ERK5 staining cells were fixed in ice-cold methanol at -20°C for 10 minutes. Cells were blocked in 1% (w/v) BSA diluted in TBST for 1hr. Primary antibody and Alexa Fluor® secondary antibodies (Invitrogen, Thermo Fisher Scientific) were diluted in 1% (w/v) BSA diluted in TBST. Images were taken on a Zeiss AxioObserver Z1 inverted fluorescence microscope with Apotome2 optical sectioning device using an X63/1.25 oil immersion objective.

Trans-endothelial permeability assay

HCMECs were plated onto ThinCerts™ 0.4 μ m translucent (12-well; Greiner, UK) and grown to confluence over 6 days. HCMECs were washed in DPBS before addition of 2 mg/ml FITC-dextran 4 kDa (Sigma, Poole, UK) diluted in phenol red free EBM

supplemented with 1% (v/v) FCS for 25 minutes at 37°C in a humidified 5% (v/v) CO₂ atmosphere, after which the fluorescence level in the flow through was measured on a Varioskan plate reader (Ex 490 nm and Em 525 nm).

Trans-endothelial electrical resistance (TEER) assay

HCMECs were plated onto ThinCerts™ 0.4 µm translucent (12-well; Greiner, UK) and grown to confluence over 6 days. Resistance was measured using a Millicell ERS-2 volttohmmeter (Millipore) and multiplied by the surface area of the insert to determine the TEER.

siRNA Transfection

SiRNA duplexes were prepared as per the manufacturer's instructions using RNAiMAX (Non silencing D-001810-10-05, MEKK2 L-003582-02-0005, MEKK3 L-003301-00-0005, MEK5 L-003966-00-0005, ERK5 L-003513-00-0005 smart pools or ERK5 J-003513-08, J-003513-10 individual duplexes, Dharmacon, GE Healthcare, UK). HCMECs were transfected with 10 nM siRNA diluted in Opti-MEM (Invitrogen, Thermo Fisher Scientific) with addition of 0.2% (v/v) Lipofectamine RNAiMAX for 6hrs. HCMECs were washed in DPBS with calcium and magnesium before addition of fresh FGM. Cells were allowed to grow to confluence for a further 6 days, with additional media changes every 2 days.

Adenoviral mediated gene expression

Constitutively active MEK5 cDNA (MEK5(D) HA-Tag) and ERK5 wild-type cDNA (ERK5 Flag-Tag) (Roberts et al., 2010) were cloned into the adenoviral vector pAd-DEST (Invitrogen). Recombinant adenovirus was generated by transient transfection of HEK293A cells and adenovirus purified using the Vivapure column (Sartorius, UK). HCMECs were infected with Ad-CA-MEK5 and Ad-ERK5 at a MOI of 10 for 24hrs. Following transfection, cells were grown in endothelial FGM until confluent.

Viability assay

HCMECs, A2780s and BT474s were plated on 96-well gelatin-coated plates and maintained at sub confluence for 24hrs. Cells were pre-incubated with 0.3 µM simvastatin before addition of doxorubicin (30-0.001 µM) for 72hrs. Cells were lysed in Cell Titer Glo® (Promega, UK) as described previously (Wilkinson et al., 2016).

Biochemical addback procedure

HCMECs were cultured as described previously before addition of Squalene (#S3626), cholesterol (#C4951), mevalonolactone (#M4667), geranylgeranyl pyrophosphate (GGPP, #G6025) and farnesyl pyrophosphate ammonium salt (FPP, #F6892) (purchased from Sigma, Poole, UK), for 24hrs.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from a range of endothelial cells and liver tissue using the RNAeasy kit following the manufacturer's instructions (Qiagen, Crawley, UK). cDNA synthesis was performed by reverse transcription of 1 µg total RNA as previously described (Wilkinson et al., 2016). Primers: *SLCO1B1* forward 5' TTGGAGCTTGGTGGCTTAAT3'; *SLCO1B1* reverse 5' CCAGCACATGCAAAGACAGT 3'. qRT-PCR was performed as described previously (Wilkinson et al., 2016). Cycle threshold (C_T) values were determined for each mRNA sample and compared to the GAPDH control to determine gene expression changes using the comparative C_T ($2^{-\Delta\Delta C_T}$) method (Livak and Schmittgen, 2001).

Statistical significance

This was performed using a one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test using SPSS software. Significant differences between control and test groups were evaluated with *P values ≤ 0.05 and **P values ≤ 0.01 , indicated on the graphs. Error bars in graphs and histograms denote \pm s.d. (standard deviation).

Results

Endothelial tight junction formation is dependent on ERK5

Mouse *ERK5* knockout studies have suggested a role for ERK5 in preserving vascular integrity (Hayashi et al., 2004). Intercellular tight junctions are critical for endothelial cell barrier formation and regulation of paracellular diffusion (Tornavaca et al., 2015). We utilised siRNA-mediated silencing of ERK5 to analyse the localisation of the tight junction protein zonula occludens-1 (ZO-1) in human cardiac microvascular endothelial cells (HCMECs). ERK5 silencing resulted in reduced localisation of ZO-1 in tight junctions (Fig. 1A) with no apparent effect on actin stress fiber formation analysed by phalloidin staining (Fig. 1A). The level of ZO-1 protein did not appear to be affected by ERK5 silencing (Fig. 1B). Loss of endothelial tight junction formation has been linked with an increase in paracellular permeability (Bazzoni and Dejana, 2004). Analysis of permeability using FITC-labelled dextran (Fig. 1C) revealed that ERK5 silencing resulted in an increase in permeability in HCMECs (Fig. 1C). We also analysed permeability by measuring transendothelial electrical resistance (TEER) (Wegener and Seebach, 2014). Treatment with ERK5 siRNA resulted in a significant decrease in TEER indicating increased permeability (Fig. 1D). Taken together, this data suggests that ERK5 regulates endothelial tight junction formation and paracellular permeability.

We have previously shown that the cardiotoxic drug doxorubicin is able to disrupt tight junction formation in HCMECs resulting in increased permeability (Wilkinson et al., 2016). We used adenoviral mediated gene transduction to express a constitutively active MEK5 (CA-MEK5) and ERK5 in HCMECs and analyse effects on tight junction formation and permeability. Expression of CA-MEK5 and ERK5 resulted in increased tight junction formation and cortical actin staining, which was able to overcome the effects of doxorubicin (Fig. 2A). Increased activation of ERK5 was confirmed by mobility shift on western blot analysis (Fig. 2B). Concomitant with the effects on tight junction formation, expression of CA-MEK5 and ERK5 also resulted in decreased permeability in HCMECs (Fig. 2C); this effect was able to prevent the increased permeability seen with doxorubicin treatment. This data shows that increased ERK5 activity stimulates tight junction formation and decreases permeability in endothelial cells.

Statins induce ERK5 activation in HCMECs

We have previously reported that VEGF is able to activate ERK5 in endothelial cells and regulate endothelial cell survival (Roberts et al., 2010). It has recently been reported that statin treatment of cells results in ERK5 activation (Chu et al., 2015; Le et al., 2014). We were interested in determining the ability of statins to activate ERK5 in HCMECs. We

initially utilised simvastatin (Zocor) to analyse effects on ERK5 and ERK1/2 in HCMECs. Treatment of cells with simvastatin for 6hrs resulted in a dose-dependent activation of ERK5 in the absence of ERK1/2 activation (Fig. 3A). To confirm the biochemical effect of statins in inhibiting HMG Co-A reductase with a concomitant reduction in cholesterol and isoprenoid synthesis, we analysed the prenylation status of the small MW GTP-binding protein Rap1A by western blotting (Antoine et al., 2010; Sidaway et al., 2004). Activation of ERK5 was only observed at time points where Rap1A was unprenylated (Fig. 3A). A number of different statins with different pharmacokinetic properties have been generated over the last 15 years. Simvastatin is a lipophilic type I statin derived from a natural product (Gazzerro et al., 2012). We also utilised another type I statin pitavastatin (Livalo) as well as rosuvastatin (Crestor) a more recently developed synthetic type II hydrophilic statin (Schachter, 2005). All statins showed dose-dependent activation of ERK5, with simvastatin the most potent with an EC_{50} of 17 nM (Fig. 3 C&D; Supplementary Fig. 1). ERK5 is activated by phosphorylation of the T-E-Y motif by the upstream kinase MEK5 (MAPKK). MEK5 is activated by phosphorylation on Ser³¹¹/Thr³¹⁵ by MEKK3 or MEKK2 (MAPKKK) (Chao et al., 1999; Sun et al., 2001). In order to determine if ERK5 was activated by simvastatin via the canonical pathway we utilised siRNA duplexes to silence expression of MEKK2, MEKK3, MEK5 and ERK5. Silencing of MEKK3 and MEK5 reduced ERK5 activation as shown by a bandshift and phosphorylation of Thr²¹⁸/Tyr²²⁰ (Fig. 3E). Silencing of MEKK2 did not affect simvastatin-mediated activation of ERK5. This data suggests that simvastatin treatment activates ERK5 in HCMECs via a pathway requiring MEKK3 and MEK5.

Statin induced ERK5 phosphorylation occurs via inhibition of protein geranylgeranylation

The previous results showed that simvastatin activation of ERK5 requires at least 6hrs pre-incubation and occurs simultaneously with the unprenylation of Rap1A. Inhibition of HMG Co-A reductase by statins leads to a reduction in mevalonate and the synthesis of isoprenoid intermediates and ultimately reduced cholesterol synthesis (Surani et al., 1983) (Fig. 4). In order to determine the biochemical pathway downstream of mevalonate reduction which leads to activation of ERK5 we used a series of biochemical add backs to determine at which point the effect of simvastatin on ERK5 activation could be prevented. Treatment with mevalonolactone, a membrane permeable ester of mevalonate (Vamvakopoulos and Green, 2003), prevented simvastatin-stimulated ERK5 activation (Fig.4B). Treatment with cholesterol and the biochemical precursor squalene did not prevent simvastatin mediated activation of ERK5 (Fig.4B). Geranylgeranyl-pyrophosphate (GGPP) and farnesyl-pyrophosphate (FPP) are two isoprenoids generated from mevalonate. Treatment with cell-permeable GGPP prevented

simvastatin mediated ERK5 activation whereas treatment with cell permeable FPP had no effect (Fig.4B). Taken together, this data suggests that simvastatin activates ERK5 by suppressing the isoprenylation of proteins via GGPP. To further confirm the role of geranylgeranylation we utilised the geranylgeranyltransferase-I inhibitor GGTI-298 (Vogt et al., 1996). This inhibitor activated ERK5 in HCMECs after 3hr preincubation (Fig. 4C). Furthermore, this inhibitor also stimulated tight junction formation (Fig.4D) and inhibited permeability in the HCMECs (Fig. 4E).

Statins stimulate endothelial tight junction formation and decreased permeability via ERK5

Our initial data showed that constitutive activation of ERK5 can lead to increased endothelial tight junction formation and decreased permeability (Fig. 2 A-C). Statins have been shown to stimulate tight junction formation in human pulmonary artery endothelial cells (Chen et al., 2014) and rat brain endothelial cells (Morofuji et al., 2010). We were interested in determining if statins were also able to stimulate tight junction formation in an ERK5 dependent manner in HCMECs. Immunofluorescence analysis of ZO-1 localisation and actin stress fibres revealed that treatment of HCMECs with simvastatin for 6hrs resulted in increased ZO-1 staining at the cell membrane and redistribution of actin stress fibres to form a cortical ring of F-actin (Fig. 5A). Preincubation with the MEK5 inhibitor BIX02189 (Tatake et al., 2008) prevented simvastatin-mediated tight junction formation and actin reorganisation (Fig. 5A). The biochemical effect of BIX02189 in preventing ERK5 activation by simvastatin was confirmed by western blotting (Fig. 5B). Furthermore, the apparent increase in ZO-1 staining at the plasma membrane following simvastatin treatment was not due to an increase in ZO-1 protein levels (Fig. 5B).

We have demonstrated that simvastatin significantly decreased endothelial permeability. This decrease in permeability was prevented by pretreatment with BIX02189 (Fig. 5C & D). This data suggests that simvastatin regulated ERK5 activity leads to increased tight junction formation and reduced permeability in HCMECs. We next investigated the potential interaction between ZO-1 and ERK5 following simvastatin treatment. Immunoprecipitation of ERK5 following simvastatin treatment for 6hrs resulted in co-precipitation of ZO-1 (Fig. 5E & F) indicating that ERK5 and ZO-1 can interact following simvastatin treatment.

Statin treatment prevents doxorubicin effects on tight junctions and cell permeability in HCMECs

Statin treatment has been shown to reduce cardiotoxicity associated with doxorubicin treatment *in vivo* using rodent models (Henninger et al., 2015). We have already shown that adenoviral mediated overexpression of ERK5 in HCMECs can stimulate tight junction formation and overcome the effects of doxorubicin in HCMECs (Fig. 2 A-C). Considering that statins are able to activate ERK5 leading to increased tight junction formation, we determined the potential for statin pretreatment to overcome the adverse effect of doxorubicin on HCMECs. Pre-treatment with simvastatin prevented doxorubicin-induced disruption in tight junction formation (Fig. 6A) and the concomitant increase in HCMEC permeability (Fig. 6C & D). Simvastatin mediated activation of ERK5 was not affected by doxorubicin treatment (Fig. 6B).

Statin treatment stimulates ERK5 translocation to the plasma membrane in HCMECs

Activation of ERK5 by phosphorylation on Thr²¹⁸/Tyr²²⁰ residues in the kinase domain is thought to result in a conformational change that disrupts a nuclear export signal allowing translocation of ERK5 to the nucleus (Gomez et al., 2016; Kondoh et al., 2006) and regulation of MEF2 mediated gene expression (Kato et al., 1997). Analysis of ERK5 translocation by immunofluorescence revealed that in HCMECs, simvastatin stimulated significant ERK5 translocation to the plasma membrane, which appeared to show a degree of co-localisation with ZO-1 (Fig. 7). This co-localisation was maintained in the presence of doxorubicin, but was prevented by the MEK5 inhibitor BIX02189 (Fig.7). Analysis of simvastatin-mediated ERK5 activation and translocation by subcellular fractionation revealed that increased ERK5 activation was evident in the membrane, cytoplasm and nuclear fractions (Fig. 8 A & B) with ERK5 showing increased translocation to the membrane (Fig. 8C).

Discussion

Endothelial tight junctions maintain vascular integrity and regulate paracellular transport of molecules. In this study we show that ERK5 regulates the tight junction adaptor protein ZO-1 in endothelial cells. Pharmacological activation of ERK5 with statins results in increased association between ZO-1 and ERK5 at the plasma membrane resulting in endothelial tight junction formation and decreased paracellular permeability. This effect can overcome both the perturbation of tight junction formation and increased permeability with doxorubicin.

The ERK5 signalling axis has been shown to play a critical role in maintaining vascular integrity in the cardiac circulation with *MEKK3/MEK5/ERK5* gene ablation in mice resulting in embryonic lethality at E9.5-10.5 (Hayashi et al., 2004; Wang et al., 2005; Yang et al., 2000). The use of siRNA mediated gene silencing of ERK5 and overexpression of ERK5 in HCMECs revealed that ERK5 regulates the tight junction associated protein ZO-1 and cell permeability (Fig. 1 & Fig. 2). This finding was further supported by the increase in permeability observed with the MEK5 inhibitor BIX02189 (Fig. 5 C & D). Gene knockout of *ZO-1* is embryonically lethal at E9.5-10.5 with embryos displaying defective angiogenesis (Katsuno et al., 2008). Recent data analysing ZO-1 function in endothelial cells has shown that ZO-1 is required for endothelial cell barrier formation and adherens junction formation (Tornavaca et al., 2015). Our data provides a novel link to explain why loss of *ERK5* results in disrupted vascular integrity and increased permeability *in vivo*.

Statins have been reported to stimulate a number of pleiotropic effects in endothelial cells independent of a reduction in serum cholesterol levels (Liao, 2005; Wolfrum et al., 2003). This vascular effect has been attributed in part to stimulation of endothelial nitric oxide synthase (eNOS) expression leading to vasodilation (Laufs et al., 1998). More recent data has shown that statin mediated activation of ERK5 in human umbilical vein endothelial cells (HUVECs) leads to induction of Kruppel-like factor 4 (KLF4) and upregulation of eNOS expression (Ohnesorge et al., 2010).

In HCMECs, analysis of the kinetics of simvastatin mediated ERK5 activation revealed that activation of ERK5 occurred simultaneously with unprenylation of Rap1A (Fig. 3). This suggests that activation occurs through inhibition of HMG Co-A reductase, the pharmacological target of statins. The ability of cell permeable GGPP to prevent simvastatin activation of ERK5 (Fig. 4B), suggested that loss of geranylgeranylation of specific GTP binding proteins, rather than loss of cholesterol synthesis, was responsible for activation of ERK5 in HCMECs. This was further confirmed by the use of the geranyltransferase I inhibitor GGTI-298 (Fig. 4C). A number of small GTP-binding proteins require prenylation with geranylgeranyl groups to localise to biological membranes and allow interaction with downstream signalling molecules. These GTP-

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binding proteins include Rho, Rac, Cdc42 and Rap (Takai et al., 2001). Recent data has shown that atorvastatin treatment of HUVECs can stimulate endothelial barrier formation and also lead to increased cytosolic localisation and activation of Rho, Rac and Cdc42 (Xiao et al., 2013). Another report has shown that simvastatin treatment of bovine aortic endothelial cells (BAECs) can lead to increased activation of Rac (Kou et al., 2009). Our data shows that statin activation of ERK5 in HCMECs requires MEKK3 (Fig. 3E). It is plausible that inhibition of membrane localisation and activation of small GTP-binding proteins leads to activation of MEKK3, MEK5 and ultimately activation and translocation of ERK5 to the plasma membrane and interaction with ZO-1 by an as yet undefined mechanism. Both simvastatin treatment of HCMECs and overexpression of constitutively active MEK5 resulted in ERK5 activation in the absence of ERK1/2 activation. Activation of ERK5 with agonists such as EGF in HeLa cells has been reported to result in a nuclear localisation of ERK5 and transcriptional regulation (Kondoh et al., 2006). In contrast, in HCMECs, simvastatin treatment led to decreased nuclear translocation of ERK5 and increased membrane translocation (Fig. 7 & Fig. 8). Statin mediated activation of ERK5, which occurs in the absence of other intracellular signalling pathways, such as ERK1/2, may allow translocation of ERK5 to the plasma membrane and regulation of tight junction formation and endothelial cell permeability.

The plasma C_{max} for simvastatin is approximately 19-30 nM (Bjorkhem-Bergman et al., 2011). Activation of ERK5 occurred at therapeutically relevant concentrations of simvastatin (Fig. 3A-C). We found that within the statin class of drugs, the more lipophilic statins simvastatin and pitavastatin were more potent activators of ERK5 than the more hydrophilic rosuvastatin. This may reflect differential uptake of the drugs into HCMECs as lipophilic statins can be absorbed by passive diffusion through cell membranes (Schachter, 2005), whereas hydrophilic statins, such as rosuvastatin, have been shown to utilise the organic anion transporters (OAT) such as OATP1B1 encoded by the *SLCO1B1* gene, which is highly expressed in hepatocytes (Niemi et al., 2011). Analysis of *SLCO1B1* mRNA expression in HCMECs revealed very low expression of these transporters compared with liver tissue (Supplementary Fig. 2). Analysis of statin uptake into different tissues in rodents has shown that the hydrophilic pravastatin inhibited sterol synthesis by 90% in the liver and ileum of rodents but less than 14% in kidney, spleen, adrenal, testis, prostate and brain, whereas the lipophilic statins lovastatin and simvastatin inhibited this process in all tissues (Koga et al., 1990).

Recent studies in mice have shown that pretreatment with statins can protect against doxorubicin-induced cardiotoxicity (Yoshida et al., 2009). This effect was attributed to direct effects on cardiomyocytes in a Rac-dependent manner. Our data would suggest that the protective effect of statins can also occur at the level of the cardiac microvasculature via activation of ERK5 and increased tight junction formation with a concomitant decrease in cellular permeability of doxorubicin ultimately reducing

exposure of cardiomyocytes to this drug. A clinical study in female breast cancer patients has shown that simultaneous use of statins and doxorubicin resulted in reduced heart failure compared with doxorubicin alone (Seicean et al., 2012). Analysis of the effect of simvastatin on viability in HCMECs exposed to doxorubicin showed a potential protective effect in contrast to enhanced toxicity to doxorubicin in A2780 ovarian cancer cells and BT474 breast cancer cells (Supplementary Fig. 3).

One potential drawback to the clinical use of statins in reducing cardiac vascular permeability and reducing cardiotoxicity is the potential reduced delivery of chemotherapeutic agent to the tumour, reducing clinical efficacy. However, an *in vivo* study in mice using three different human tumour xenografts has shown that lovastatin potentiates doxorubicin anti-tumour activity whilst reducing doxorubicin-associated cardiotoxicity (Feleszko et al., 2000). This would appear to back up our *in vitro* data. Furthermore, it is possible that statin mediated effects in potentially reducing tumour vascular permeability and delivery of drug to the tumour are ameliorated due to the inherent leakiness of tumour blood vessels compared with normal vasculature (Azzi et al., 2013; De Bock et al., 2011).

In summary, our data shows that ERK5 regulates tight junction formation and permeability in cardiac microvascular endothelial cells. Statin mediated ERK5 activation results in the translocation of ERK5 to the plasma membrane and regulation of tight junction formation and decreased endothelial cell permeability. Activation of ERK5 by drugs such as statins may have therapeutic potential in alleviating conditions where endothelial cell barrier formation is compromised such as drug-induced cardiac injury (Wilkinson et al., 2016), arterial hypertension (Laine, 1988), sepsis (Rachoin et al., 2013) and diabetic nephropathy (Peng et al., 2013).

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Conflict of Interest. None declared

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Figure Legends

Fig. 1. siRNA mediated gene silencing of ERK5 induced HCMEC barrier perturbation. HCMEC were transfected with non-silencing or ERK5 siRNA for 6hrs and allowed to reach confluence over 5 subsequent days. (A) Immunofluorescence imaging of tight junctions (ZO-1, green), actin stress fibres (phalloidin, red) and nuclei (Hoechst, blue). Arrows indicate barrier perturbation. Scale bars: 10 μ m. Results are from 1 experiment representative of 3. (B) Western blot of ZO-1, ERK5, ERK1/2 and GAPDH levels in HCMECs following siRNA induced ERK5 gene silencing. (C) HCMECs were plated on Thincerts™ containing 0.4 μ m pores and permeability of 4 kDa FITC-dextran across the HCMEC monolayer was assessed and compared to untransfected cells, (n=4), mean \pm s.d. **P \leq 0.01 compared to untransfected. (D) Assessment of TEER was conducted on HCMECs plated on Thincerts™ containing 0.4 μ m pores, (n=4), mean \pm s.d. **P \leq 0.01 compared to untransfected.

Fig. 2. Adenoviral mediated ERK5 expression stimulates HCMEC tight junction formation. HCMEC were transfected with Ad-Control or Ad-CA-MEK5 and Ad-ERK5 for 24 hours before treatment with doxorubicin 0.1 μ M for 6hrs. (A) Immunofluorescence imaging of HCMEC tight junctions (ZO-1, green), actin stress fibres (phalloidin, red) and nuclei (Hoechst, blue). Arrows indicate barrier perturbation. Scale bars: 10 μ m. Results are from 1 experiment representative of 3. (B) Western blot of ERK5, ERK1/2, phospho ERK1/2 and GAPDH levels in HCMECs. FLAG-Tag was used to confirm transfection of Ad-ERK5 and HA-Tag was used to confirm transfection of Ad-CA-MEK5. (C) HCMECs were plated on Thincerts™ with 0.4 μ m pores and permeability of 4 kDa FITC-dextran across the HCMEC monolayer assessed and compared to Ad-Control (n=4), mean \pm s.d. *P \leq 0.05 compared to Ad-Control.

Fig. 3. Simvastatin stimulates ERK5 phosphorylation in HCMECs. (A) HCMECs were incubated with simvastatin at a range of concentrations for 6hrs. Intracellular signalling responses were assessed by western blotting for phosphorylation of ERK5 and ERK1/2 using phospho-specific antibodies. Unphosphorylation of Rap1A was measured as a control for simvastatin activity. Total protein was measured for ERK5, ERK1/2 and Rap1. (B) HCMECs were incubated with 0.3 μ M simvastatin at a range of time points. Intracellular signalling responses were assessed by western blotting for phosphorylation of ERK5 and ERK1/2 using phospho-specific antibodies. Unphosphorylation of Rap1 was measured as a control for simvastatin activity. Total protein was measured for ERK5, ERK1/2 and Rap1. (C) The level of ERK5 band shift following treatment with simvastatin, rosuvastatin and pitavastatin was quantified relative to vehicle control. (D) Activation of

ERK5 by band shift was determined (EC_{50} concentration), mean \pm s.d. (n=3). (E) HCMEC were transfected with siRNA to MEKK2, MEKK3, MEK5 and ERK5 for 6hrs and allowed to reach confluence over 5 subsequent days before treatment with 0.1% DMSO or 0.3 μ M simvastatin for 6hrs. Western blot of ERK5, phosphorylated-ERK5, MEKK2, MEKK3, MEK5 and GAPDH levels in HCMEC. (F) Quantification of ERK5 band shift (n=3) mean \pm s.d. * $P \leq 0.05$ compared to untransfected basal.

Fig. 4. Statin induced ERK5 phosphorylation occurs via inhibition of protein geranylgeranylation

(A) Summary of the key substrates in the cholesterol biosynthesis pathway. HCMECs were treated with (B) mevalonolactone 50 μ M, GGPP 10 μ M, FPP 10 μ M, squalene 10 μ M or cholesterol 10 μ M for 24hrs in the presence and absence of simvastatin (0.3 μ M for 6hrs). ERK5 expression was assessed by western blotting. Unprenylation of Rap1A was measured as a control for simvastatin activity. The level of ERK5 activation assessed by mobility band shift was quantified and expressed as fold change relative to vehicle control (n=4) mean \pm s.d. * $P \leq 0.05$, ** $P \leq 0.01$ compared to vehicle control basal. (C) HCMECs were treated with GGTI-298 (10 μ M) for a range of time points. Intracellular signalling responses were assessed by western blotting for ERK5, unprenylated Rap1A and Rap1. (D) Immunofluorescence imaging of HCMEC tight junctions (ZO-1, green), actin stress fibres (phalloidin, red) and nuclei (Hoechst, Blue) following treatment with GGTI-298 (10 μ M for 6hrs). Scale bars: 10 μ m. (E) HCMEC were plated on Thincerts™ with 0.4 μ m pores and permeability of 4 kDa FITC-dextran across the HCMEC monolayer was assessed, (n=4), mean \pm s.d. * $P \leq 0.05$ compared to vehicle control.

Fig. 5. MEK5 inhibition prevents simvastatin induced tight junction formation and ERK5 phosphorylation.

HCMECs were pre-incubated with BIX02189 1 μ M for 30 minutes prior to addition of simvastatin 0.3 μ M for 6hrs. (A) Immunofluorescence imaging of HCMEC tight junctions (ZO-1, green), actin stress fibres (phalloidin, red) and nuclei (Hoechst, blue). Scale bars: 10 μ m. Results are from 1 experiment representative of 3. (B) Western blot of ZO-1, ERK5, phospho ERK5, unprenylated Rap1A and GAPDH levels in HCMECs. Level of ERK5 phosphorylation is quantified relative to vehicle control. Mean \pm s.d. (n=3) * $P \leq 0.05$ compared to vehicle control basal. (C) Permeability of 4 kDa FITC-dextran through an endothelial monolayer on ThinCerts™, (n=4), mean \pm s.d. * $P \leq 0.05$, ** $P \leq 0.01$ compared to vehicle control. (D) Assessment of TEER was conducted on HCMECs plated on Thincerts™ containing 0.4 μ m pores, (n=4), mean \pm s.d. ** $P \leq 0.01$ compared to vehicle control. (E) HCMECs were treated with simvastatin 0.3 μ M for 6hrs before immunoprecipitation with IgG or ERK5 antibodies. Western blot of ZO-1 following immunoprecipitation with ERK5 in HCMECs. (F) Level of ZO-1 and ERK5 is quantified

relative to vehicle control in IP lysates. Mean \pm s.d. (n=3). **P \leq 0.01, compared to vehicle control.

Fig. 6. Simvastatin prevents doxorubicin induced barrier perturbation. HCMEC were pre-incubated with simvastatin 0.3 μ M for 6hrs before treatment with doxorubicin 0.1 μ M for a further 6hrs. (A) Immunofluorescence imaging of HCMEC tight junctions (ZO-1, green), actin stress fibres (phalloidin, red) and nuclei (Hoechst, blue). Scale bars: 10 μ m. Results are from 1 experiment representative of 3. (B) Western blot of ZO-1, ERK5, ERK1/2, unphosphorylated Rap1A and Rap1 levels in HCMECs. Level of ERK5 activation is quantified relative to vehicle control. Mean \pm s.d. (n=3) **P \leq 0.01 compared to vehicle control. (C) HCMEC were plated on Thincerts™ with 0.4 μ m pores and permeability of 4 kDa FITC-dextran across the HCMEC monolayer was assessed and compared to vehicle control, (n=4), mean \pm s.d. *P \leq 0.05, **P \leq 0.01 compared to vehicle control. (D) Assessment of TEER was conducted on HCMECs plated on Thincerts™ with 0.4 μ m pores, (n=4), mean \pm s.d. *P \leq 0.05, **P \leq 0.01 compared to vehicle control.

Fig. 7. ERK5 and ZO-1 colocalise following simvastatin treatment in HCMECs. Immunofluorescence imaging of HCMEC tight junctions (ZO-1, green), ERK5 (red) and nuclei (Hoechst, blue) following treatment with simvastatin 0.3 μ M and/or doxorubicin 0.1 μ M or BIX02189 1 μ M for 6hrs. Scale bars: 10 μ m. Arrows indicate co-localisation. Results are from 1 experiment representative of 3.

Fig. 8. ERK5 cellular translocation following simvastatin treatment in HCMECs. HCMECs were treated with simvastatin 0.3 μ M for 6hrs before subcellular fractionation performed. (A) Western blot of ZO-1 and ERK5. ATP1A1, actin and lamin B were used as positive controls to show cellular fraction specificity in membrane, cytoplasm and nuclear fractions respectively. Whole cell lysate (WCL). (B) Quantification of the total ERK5 level in each compartment (n=3), mean \pm s.d. *P \leq 0.05 compared to vehicle control for each fraction. (C) Quantification of ERK5 band shift relative to each fraction's vehicle control (n=3), mean \pm s.d. *P \leq 0.05 compared to vehicle control for each fraction.

Supplementary Fig. Legends

Supplementary Fig. 1. Statin induced ERK5 phosphorylation. HCMECs were incubated with (A) rosuvastatin and (B) pitavastatin at a range of concentrations for 6hrs. ERK5 phosphorylation was assessed by mobility band shift on western blotting. Unprenylation of Rap1A was measured as a control for statin activity.

Supplementary Fig. 2. Transporter expression in endothelial cells in comparison to the liver. Total RNA was isolated from HCMECs #1-3, HDMECs 1 & 2, HCAEC 1 & 2, HBMEC, HUVEC, Liver 1 & 2 and mRNA levels were analysed by qRT-PCR for expression of *SLCO1B1*. (n=3), mean \pm s.d.

Supplementary Fig. 3. Cell viability in response to doxorubicin in the presence and absence of simvastatin. (A) HCMECs, (B) A2780s and (C) BT474s were treated with doxorubicin alone and in the presence of simvastatin 0.3 μ M for 72hrs. Cell viability was measured by Cell Titer Glo ATP assay. (D) IC₅₀ levels were determined, mean \pm s.d. (n=4), *P \leq 0.05 compared to doxorubicin alone, one-way ANOVA, SPSS.

Figure 1

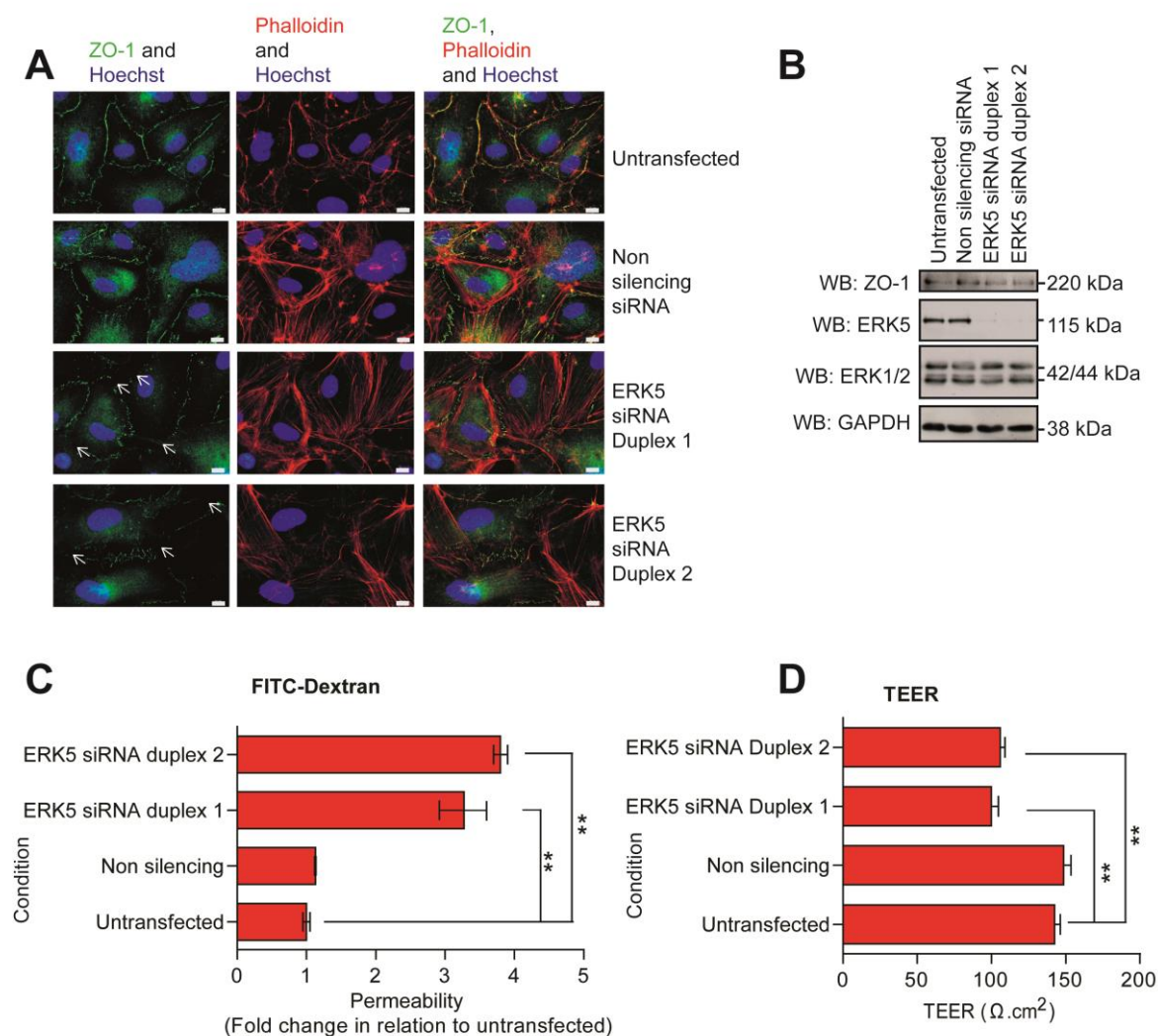


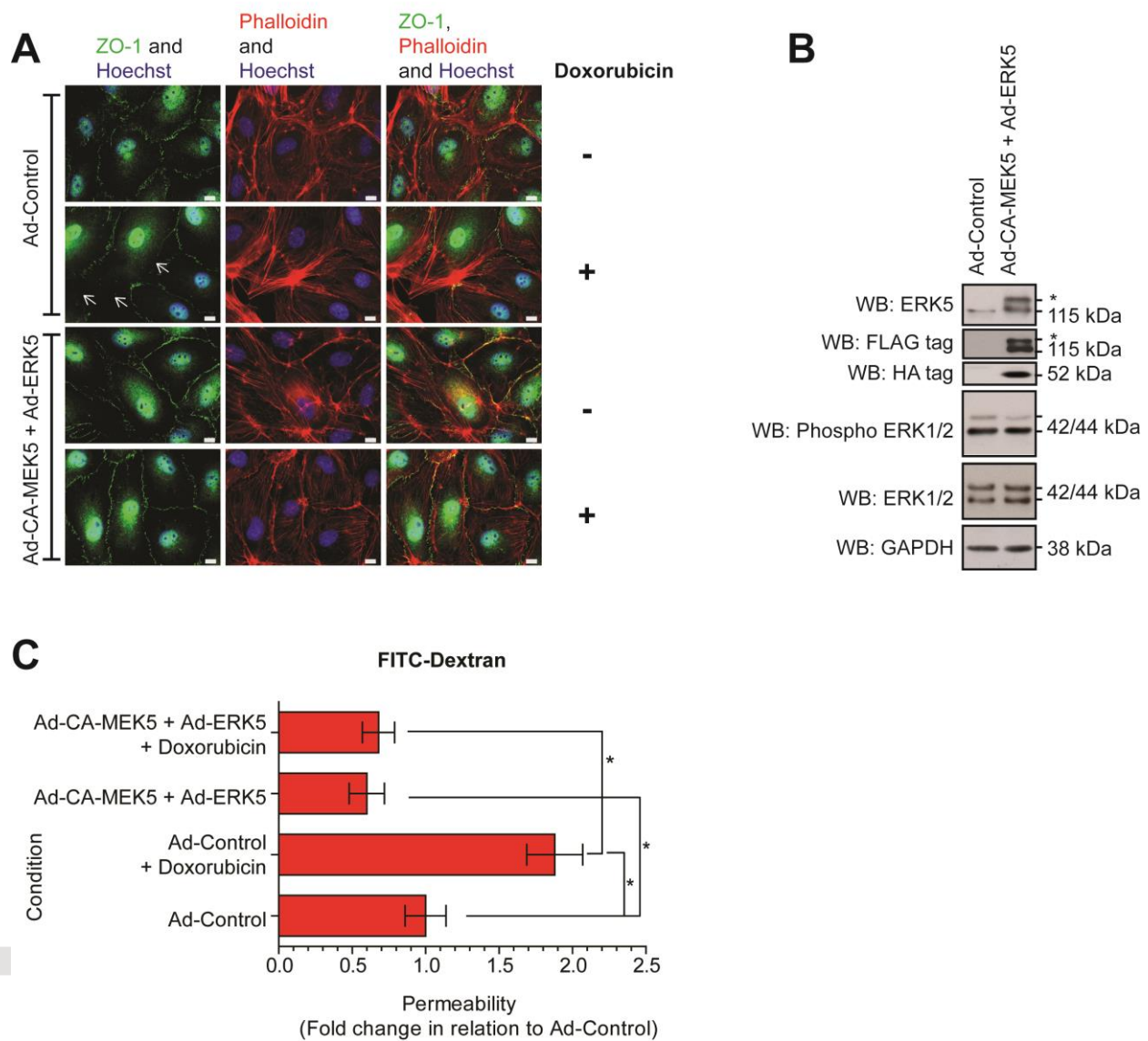
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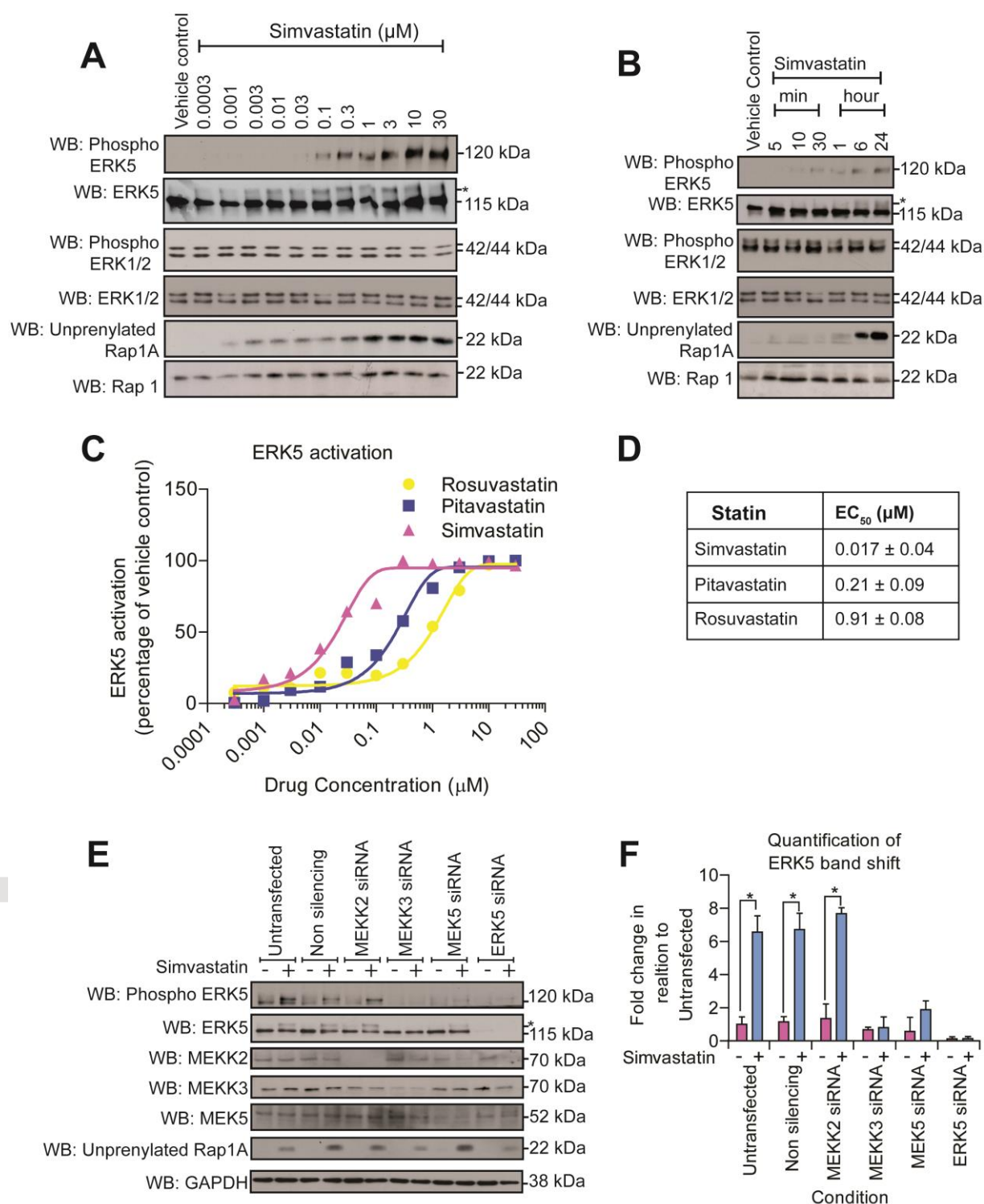
Figure 3

Figure 4

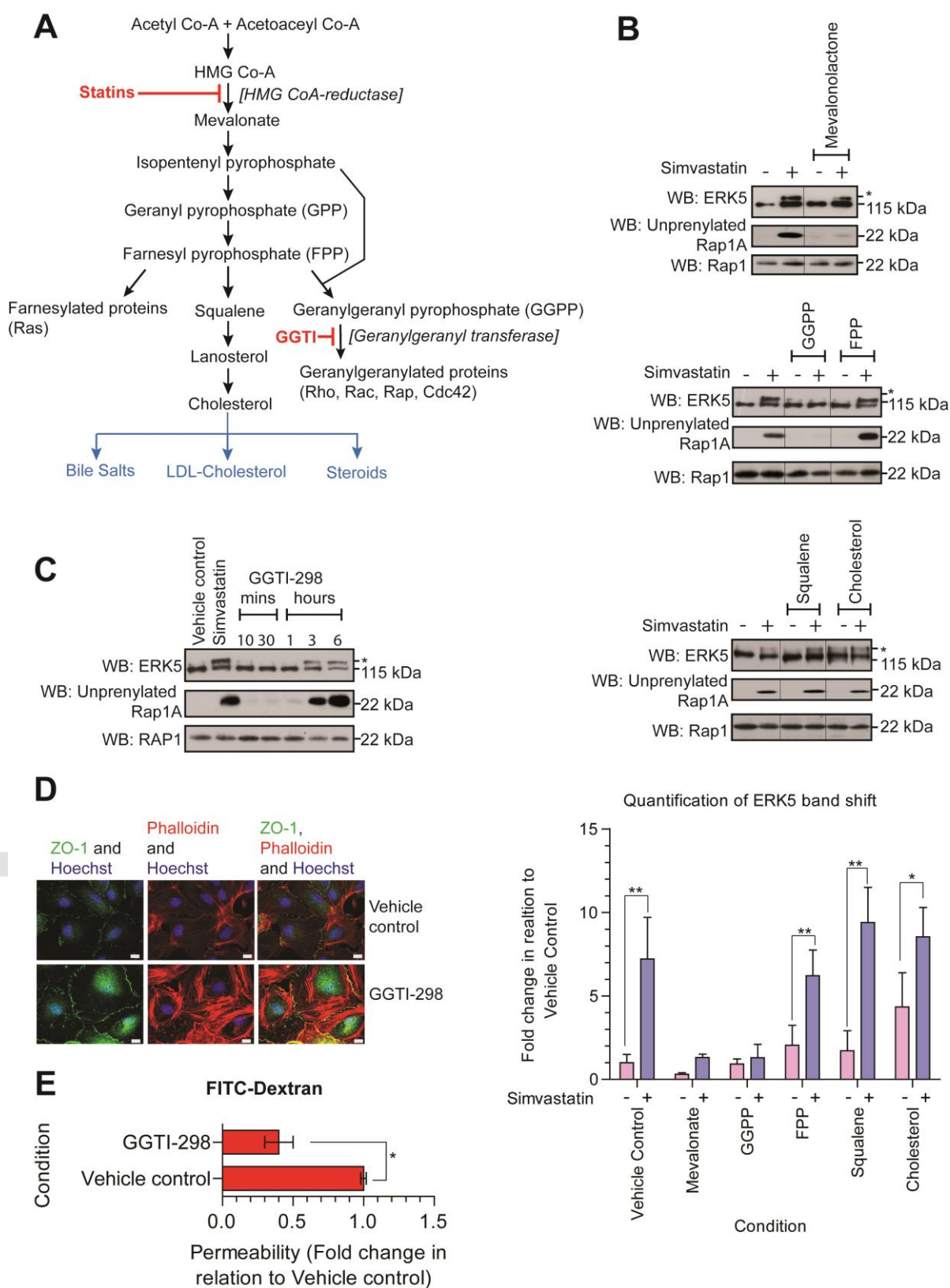


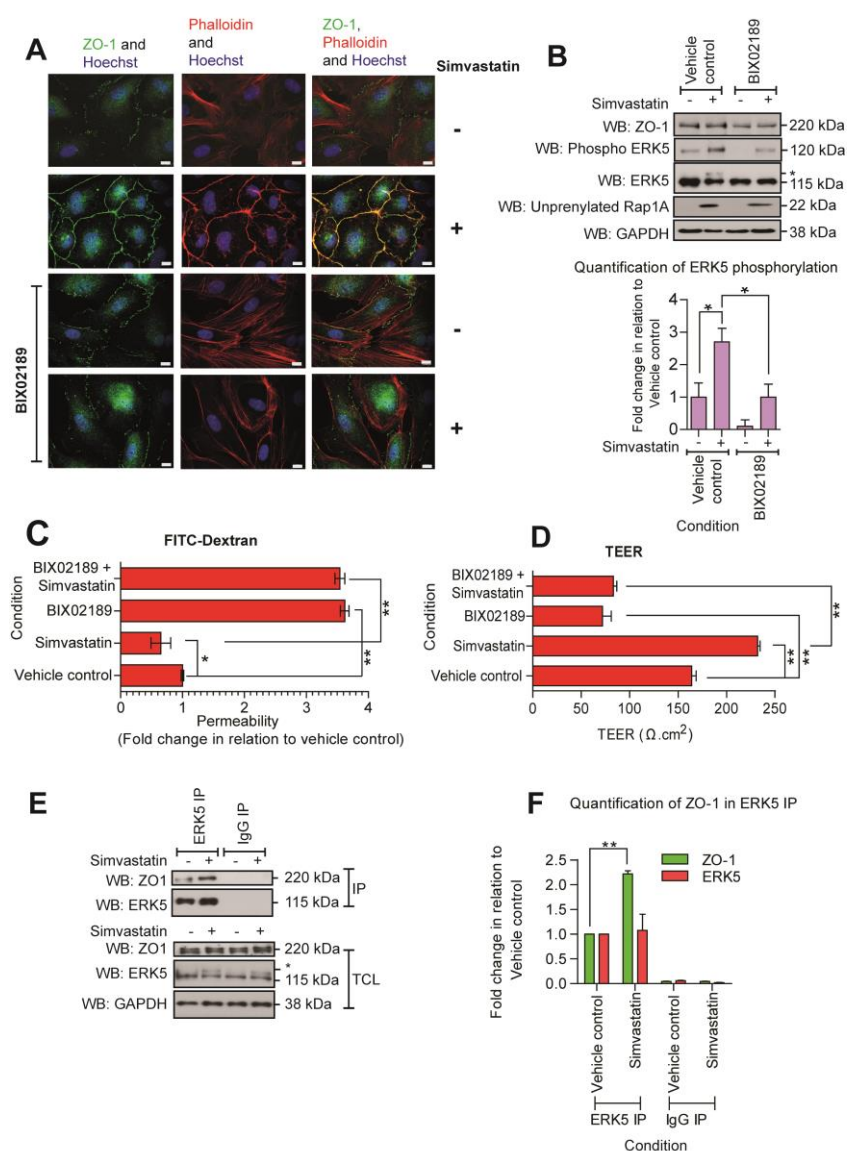
Figure 5

Figure 6

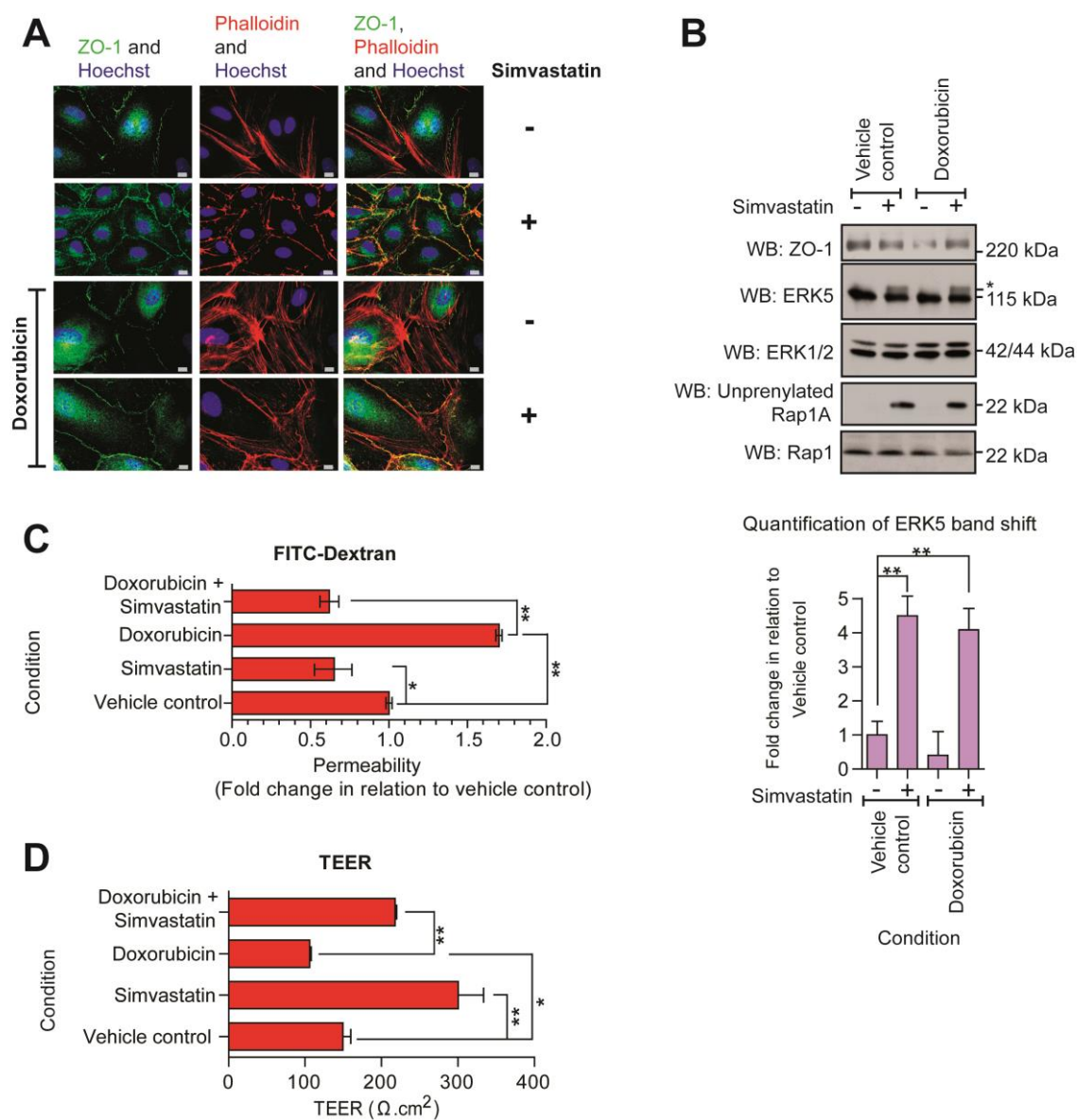


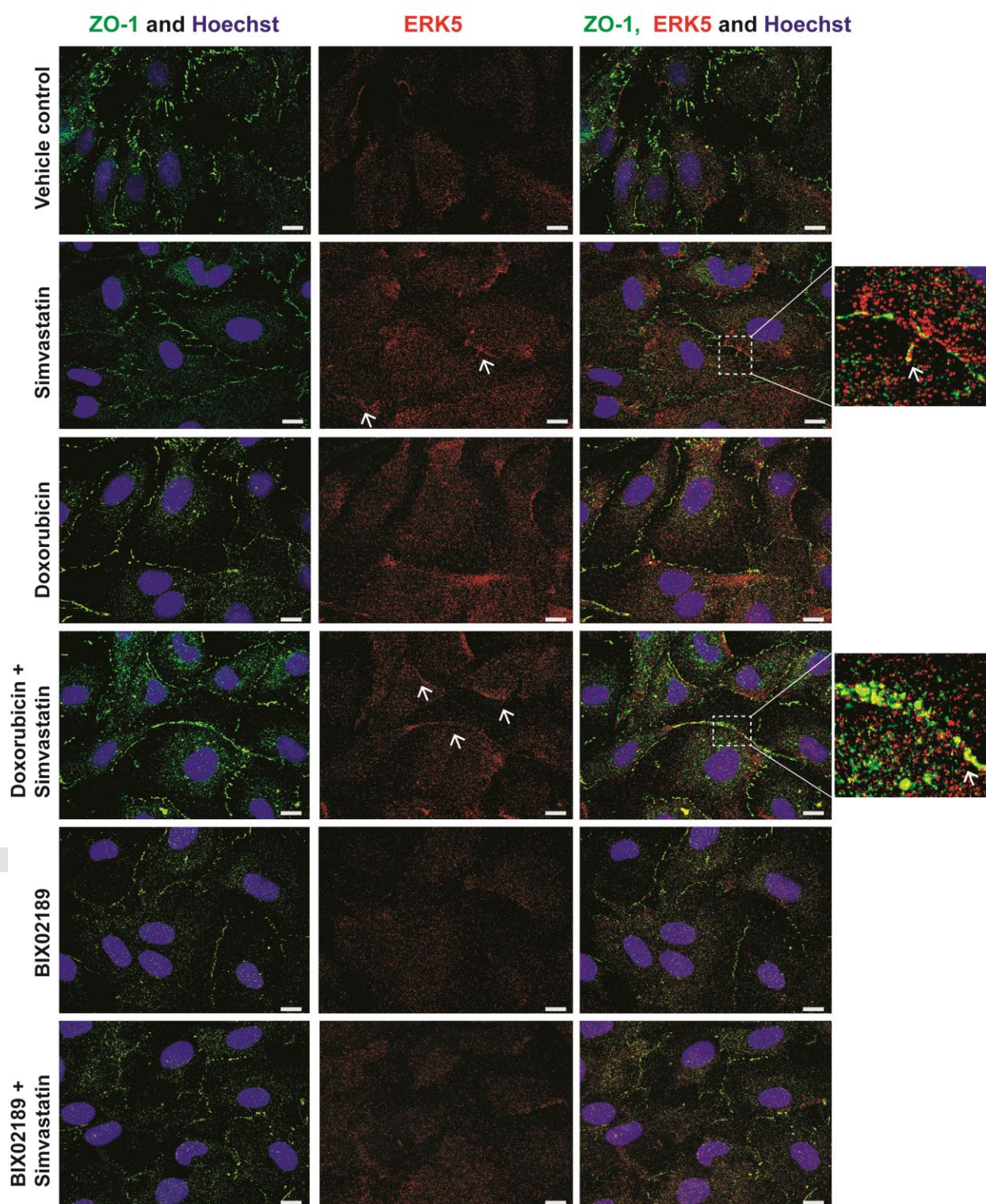
Figure 7

Figure 8